High specific activity for anammox bacteria enriched from activated sludge at 10°C

Tim L.G. Hendrickx[a,*], Christel Kampman[a], Grietje Zeeman[a], Hardy Temmink[a], Ziye Hu[b], Boran Kartal[b] and Cees J.N. Buisman[a]

[a] Sub-department of Environmental Technology, Wageningen University, P.O. Box 17, 6700 AA Wageningen, The Netherlands

[b] Department of Microbiology, IWWR, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

* corresponding author: tel. +31 317 48 33 39; fax: +31 317 48 21 08.

E-mail address: tim.hendrickx@wur.nl (Tim Hendrickx)

Abstract

Anammox in the water line of a waste water treatment plant (WWTP) saves energy for aeration and allows for recovering biogas from organic material. Main challenges for applying the anammox process in the water line are related to the low temperature of <20 °C, causing a significant drop in the specific anammox activity. The aim of this research was to enrich a cold-adapted anammox species, with a high specific activity. This was achieved in a 4.2 L reactor operated at 10 °C, fed with 61 mg (NH_4 + NO_2)-N/L and inoculated with activated sludge from two selected municipal WWTPs. *Candidatus Brocadia fulgida* was the dominant species in the enriched biomass, with a specific activity was 30–44 mg N/(g VS d). This is two times higher than previously reported at 10 °C, which is beneficial for full scale application. Biomass yield was 0.046 g biomass/g N converted, similar to that at higher temperatures.

Keywords

Autotrophic nitrogen removal, 10°C anammox enrichment, specific activity, low ammonium concentration, temperature dependency

1. Introduction

In municipal and industrial waste water treatment, the anammox process is widely applied for treatment of streams with a high ammonium concentration (Hu et al., 2013b). Anaerobic ammonium oxidising (anammox) bacteria oxidise ammonium autotrophically to dinitrogen gas, using nitrite as a terminal electron acceptor. This process has clear advantages over conventional nitrogen removal by nitrification and denitrification, such as lower aeration costs and no need for an (external) carbon source for denitrification. An example of full scale application of the anammox process is the treatment of reject water from anaerobic sludge digestion at municipal waste water treatment plants (WWTPs) (Abma et al., 2007). Separate treatment of the reject water alleviates the internal nitrogen load on the water line of the WWTP to which it is fed. However, the bulk of the nitrogen (>80%) still needs to be removed from the water line via nitrification–denitrification at the expense of biologically degradable organic material. Autotrophic nitrogen removal in the water line with anammox bacteria would result in a significant reduction in aeration costs and the organic material present in the waste water will no longer be required for denitrification. Instead, it could be used for biogas production in, for example, a direct anaerobic treatment step prior to nitrogen removal (Zhang et al., 2012) or by enhanced pre-sedimentation followed by anaerobic digestion of this primary sludge (Kartal et al., 2010, Siegrist et al., 2008). In moderate climates, the application of anammox in the water line faces several challenges, which are mostly related to the low waste water temperature of 5–20 °C. Current full scale anammox processes operate at mesophilic temperatures and

the optimum temperature of the anammox bacteria from these reactors has been found to lie between 30 and 37 °C (Dosta et al., 2008, Strous et al., 1999). Consequently, lowering the temperature from 35 to 20 °C resulted in a large decrease in biomass specific activity (Dosta et al., 2008, Vázquez-Pádin et al., 2011). Accordingly, a much higher biomass concentration or a larger reactor volume would be required to treat the waste water at a lower temperature. Other challenges of application of anammox in the water line are the high flow rates and the low ammonium concentrations, typically 20–70 mg N/L (Metcalf and Eddy, 2004). The low N concentrations itself should not pose a problem, given the high affinity of anammox bacteria for both NO_2^- and NH_4^+ (<0.07 mg N/L, Strous et al., 1999) and this was also confirmed at 20 °C by Hendrickx et al. (2012). The high flow rate, however, requires reactors that need to be operated at short hydraulic retention times, to obtain an acceptable reactor volume. This will require very good biomass retention and/or biomass with a high specific activity. The latter may be achieved by adaptation of anammox biomass to lower temperatures, as was shown to be feasible at temperatures down to 15 °C by Dosta et al. (2008) and 12 °C by Hu et al., 2013a, Hu et al., 2013b. Anammox species with a lower temperature optimum of 15 °C or even 12 °C have been shown to exist (Dalsgaard and Thamdrup, 2002, Rysgaard et al., 2004). These were determined for anammox bacteria from marine sediments which had very low environmental temperature of, respectively, 4–6 °C and −1.7 to 4 °C. When marine anammox species (Scalindua) were enriched in lab reactors operated at a higher temperature of 15 or 23 °C, a higher temperature optimum of 25–30 °C was found (van de Vossenberg et al., 2008), showing that operational or environmental temperature may at least partially determine the optimum temperature. Enrichment of anammox at low temperatures (<18 °C) without inoculation with a large amount of anammox biomass has not been shown previously, mainly because focus has been on application of the

anammox process for warm and concentrated waste waters. With the recent developments towards more energy efficient treatment of municipal waste water, low temperature application of anammox becomes very interesting. Few anammox reactors have been operated with the combination of a low nitrogen concentration (<100 mg N/L) and a temperature ⩽20 °C (see Table 1) and shown that biomass growth (and thus an increase in activity) is feasible under these conditions. In addition to the challenges related to the anammox bacteria, the production of nitrite by ammonium oxidising bacteria (AOB) as a substrate for anammox bacteria is another challenge at low temperature. Growth of AOB at low temperature is already achieved in the activated sludge process at municipal WWTPs. However, activated sludge processes also contain nitrite oxidising bacteria (NOB), which should not be present when anammox is applied in the water line. The competition between anammox bacteria and NOB for nitrite and between AOB and NOB for oxygen are additional challenges for application of low temperature anammox, but are not investigated in this paper.

This paper describes the enrichment of anammox bacteria at 10 °C, the lowest temperature reported so far, and a low influent nitrogen concentration of 61 mg $(NH_4^+ + NO_2^-)$-N/L. The aim was to selectively enrich an anammox species that may be adapted to a low temperature, which, therefore, has a high specific activity. A suitable inoculum was selected after molecular analysis for the abundance and diversity of anammox bacteria in sludge samples from 9 different WWTPs. The activity of the enriched anammox biomass was determined at temperatures between 5 and 30 °C. Moreover, the most abundant anammox bacteria was determined by molecular analyses and the copy numbers of the anammox bacteria in the enrichment culture were determined with quantitative PCR (qPCR). The advantages of low temperature enriched anammox

100 biomass for application in the water line of municipal waste water treatment will be
101 discussed.

102 **Table 1** Lab scale anammox reactors operated at T ≤ 20°C and influent nitrogen
103 concentration < 100 mg N/L. NRR = nitrogen removal rate. RBC = rotating biological
104 contactor, UASB = upflow anaerobic sludge blanket, SBR = sequencing batch reactor,
105 MBR = membrane bioreactor

	T (°C)	Influent (mg N/L)	Reactor type	including nitritation	NRR (g N/L/d)	Comments
Hendrickx et al., 2012	20	69	gaslift	-	0.29	25°C anammox sludge as inoculum
Osaka et al., 2012	18	20-50	Upflow column with non-woven	-	0.15-0.20	river sediments as inoculum
Ma et al., 2013	16	51	UASB-like	-	3.5	startup at 30°C, days 137-200 at 16°C
De Clippeleir et al., 2013	14	50	RBC	+	0.60	start-up at 29°C
Hu et al., 2013a,b	12	70	SBR	+	0.028	start-up at 30°C
this study	10	61	SBR/MBR	-	0.027	activated sludge mixture as inoculum

106

107 ## 2. Materials and Methods

108 *2.1 Inoculum selection*

109 Nine municipal waste water treatment plants (WWTPs) were selected for sampling based
110 on a low ratio of biological oxygen demand and total nitrogen (BOD/N) and a long sludge
111 retention time (SRT), as shown in Table 2 and also in Luesken et al. (2011). The Haarlo
112 WWTP was an exception to these selection criteria, but was included due to its low N
113 removal efficiency (~50%) and relatively high effluent N (29 mg N/L). Sampling of

activated sludge was performed at the end of the winter period to increase the chance of finding anammox species that were already adapted to lower temperatures. Samples were stored in closed containers at 5 °C until the reactor was inoculated. At the time of inoculation, the sludge samples contained 0.01–0.02 mg NO_2-N/ and 0.3–0.5 mg NO_3-N/L showing that the sludge had not become anaerobic.

Table 2 Characteristics of the nine waste water treatment plants that were sampled. N.a. = not available.

#	WWTP	BOD/N	SRT (d)
1	Kralingseveer	n.a.	n.a.
2	Houten	3.5	29
3	Driebergen	3.3	28
4	Zutphen	2.8	25
5	Haarlo	4.2	8
6	Varsseveld	4.7	24
7	Lichtenvoorde	2.5	26
8	Dordrecht	n.a.	n.a.
9	Heerenveen	3.7	28

2.2 Influent composition

Synthetic medium was used, containing $NaNO_2$ (30 mg N/L), NH_4Cl (30 mg N/L), $NaNO_3$ (0–14 mg N/L), $KHCO_3$ (0.4 g/L), KH_2PO_4 (0.040 g/L), $MgSO_4 \cdot 7H_2O$ (0.25 g/L) and $CaCl_2$ (0.30 g/L) and micro-nutrients (as described in van de Graaf et al., 1996) dissolved in demi water. After 189 days of operation 10% (v/v) of stabilised and filtered (0.45 μm) anaerobic effluent was added as a potential source of other micronutrients, as described in Hendrickx et al. (2012). The anaerobic effluent originated from a UASB-digester system treating domestic waste water as described in Zhang et al. (2012) and contained 40–80 mg NH_4-N/L and 80–100 mg COD/L. The heterotrophic denitrification

capacity of the stabilised and filtered UASB effluent was determined at <1 mg NO_2-N/L, as reported in Hendrickx et al. (2012).

2.3 Reactor operation

The 4.2 L gas-lift reactor used for the enrichment is schematically shown in Fig. 1. Reactor temperature was maintained at 10 °C using a thermostatic water bath. Temperature (Pt100) and pH (PHM201, Radiometer Analytical) in the reactor were measured and recorded. Initially gas recirculation over the reactors was applied for mixing. This was stopped after 195 days, after which N_2 gas (1 L/min) and CO_2 gas (1.5–3.0 mL/min) were continuously fed to the reactor without recirculation. Reactor pH was maintained at 7.5 by adjusting the CO_2 gas flow rate. Operation of the reactor could be divided into 4 phases:

I. Day 1–181: decreasing in heterotrophic denitrifying activity; operation as sequencing batch reactor (SBR);

II. Day 181–363: gradual increase in anammox activity, operation as SBR;

III. Day 363–622: increase in anammox activity, operation as SBR with membrane for full biomass retention;

IV. Day 622–722: strong increase in anammox activity, continuous operation with membrane.

During operation as an SBR a liquid replacement volume of 0.15–1 L/d was applied in combination with a settling time of 1 h. During phase I, additional biomass retention was achieved by centrifuging (10 min, 4500 rpm) 60–90% of the effluent from the enrichment reactors and returning the sludge pellet to the reactor. Initial operation as an SBR was chosen to allow selection of a specific anammox type, as it gradually washes out other,

slower growing, anammox bacteria. Once this had occurred (observed by an increase in nitrogen removal rate), full sludge retention was applied by placing a membrane in the reactor (day 363). This tubular polyvinylidene fluoride (PVDF) membrane had a pore size of 250 kDa. From day 622 onwards the reactor was continuously fed with a flow of 1–2.3 L/d. Influent entered via the top of the reactor, using a Stepdos 03 RC membrane pump. For the effluent (and later permeate) a Watson Marlow 403U peristaltic pump was used. To simulate the conditions of effluent from anaerobic reactors, methane gas was dosed to the reactors to obtain a dissolved methane concentration of ±15 mg/L; this methane dosing was stopped after 136 days.

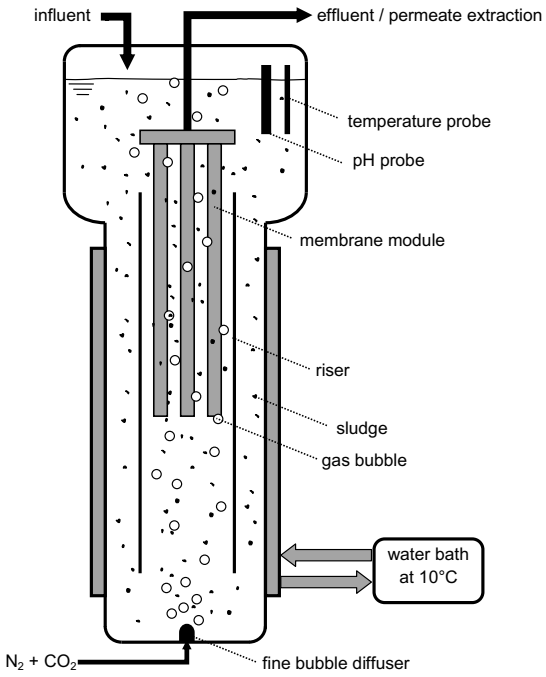


Figure 1 Scheme of the 4.2 L enrichment reactor.

2.4 Reactor measurements

Nitrite concentration was frequently determined using test strips in a 2 mL sample from the reactor; nitrogen load was increased when nitrite concentration was below 1 mg N/L. Conversion ratios (NO_2/NH_4 and NO_3/NH_4) and volumetric activity (mg N/(L d)) were

occasionally determined in a 1–3 day test. For this purpose the concentrations of NH_4, NO_2 and NO_3 in the supplied influent and in the reactor (at the start and at the end) were measured in centrifuged samples (10 min at 10,000 rpm).

Activity measurements at different temperatures were performed with the whole reactor, by decreasing the temperature of the water bath for a period of 1–3 days. At each temperature, the conversion ratios and volumetric activity were calculated.

Conversion of nitrogen species (NO_2^-, NO_3^- and NH_4^+) was calculated using the following equation: N converted (mg) = influent volume × $([N]_{in} - [N]_{out}) + V_{reactor} \times ([N]_{R,start} - [N]_{R,end})$; with [N] the N-species concentration in the influent (in), effluent (out) and reactor (R, at start and end of the test).

Net growth rate was calculated as μ = ln (activity t_1/activity t_2)/($t_1 - t_2$).

2.5 Batch activity measurements

In addition to temperature experiments with the whole reactor, batch activity experiments were performed with biomass from the reactor. Batch bottles (120 mL) with 75 mL of mixed reactor liquid and 10–15 mL of synthetic medium were used, to achieve initial concentrations of 5.3–15.9 mg NH_4-N/L and 3.6–16.2 mg NO_2-N/L. Bottles flushed with a N_2/CO_2 gas mixture (0.15% CO_2) and sealed with a rubber stopper. The bottles were placed on shakers in temperature-controlled cabinets (at 5, 7.4, 10, 15, 17, 20 and 30 °C). Concentrations of ammonium, nitrate and nitrite were measured after certain time intervals. Activities were calculated in mg N/($L_{reactor}$ d).

2.6 Analytical

Nitrite concentrations were estimated using test strips (Merckoquant). Nitrate, nitrite and ammonium were determined colorimetrically according to Standard Methods

198 (APHA, 1998) using Dr. Lange® test kits. Prior to analysis, solids were removed from
199 the samples by centrifugation at 10,000 rpm for 10 min. Total and volatile (suspended)
200 solids, TS(S) and VS(S), were measured according to Standard Methods (APHA, 1998)
201 using Whatman grade 40 filter papers (pore size 8 mm) for the TSS/VSS measurements.
202 TS and VS measurements were used for the sludge concentration in the reactor after the
203 membrane was placed (day 363).
204
205 *2.7 DNA extraction, PCR, cloning and q-PCR analysis*
206 Genomic DNA was extracted from 1 ml of sludge sample directly taken from the waste
207 water treatment plants as previously described (Schmidt et al., 1991). The 16S rRNA
208 genes were amplified by PCR using anammox specific forward primer pla46F
209 (Juretschko et al., 1998) and universal reverse primer 1529R (Neef et al., 1998). The
210 amplicons were then cloned and sequenced as previously described (Hu et al., 2013a,
211 Hu et al., 2013b). Phylogenetic trees were constructed in MEGA5 software (Tamura et
212 al., 2011). For qPCR, primers set hzsA_1597F/hzsA_1859R (Harhangi et al., 2012)
213 targeting anammox functional gene hydrazine synthase (*hzsA*) were used. The qPCR
214 reactions were performed as described previously (Hu et al., 2013a, Hu et al., 2013b). In
215 short, *hzsA* gene was first PCR amplified and cloned to create plasmids containing the
216 target gene. Extracted plasmids were quantified on a NanoDrop 1100 spectrophotometer
217 (Thermo Scientific, USA) and then serially diluted in 10-fold steps for the standard
218 curve construction. Each qPCR reaction was performed triplicate on a MyiQTM Single-
219 Color Real-Time PCR Detection System (BIO-RAD, USA). Sequences are submitted
220 and NCBI accession number will be available soon.
221
222

3. Results and Discussion

3.1 Presence of anammox at municipal WWTPs

Anammox bacteria were present in all 9 WWTP sludge samples, as revealed by clone libraries constructed with primers targeting the 16S rRNA gene of anammox bacteria. Species similar to *Brocadia*, *Kuenenia* and *Scalindua* genera were detected, as shown in the phylogenetic tree in Fig. 2. Apparently, anammox bacteria can survive under aerated conditions in the water line of a WWTP, even after a long period of low water temperature, as samples were taken at the end of the winter period. Given the low fractions of anammox bacteria in all the WWTP sludge samples, they probably did not contribute significantly towards nitrogen removal at the WWTPs. The presence of anammox bacteria in sludge from the Lichtenvoorde WWTP may also be explained by the industrial anammox reactor at Hulshof tanneries (Frijters et al., 2007). This reactor discharges its effluent, with (some) washed out anammox bacteria, to the Lichtenvoorde WWTP, which made it highly likely that this sludge would contain more viable anammox cells. The Lichtenvoorde sludge sample was chosen as the inoculum in this study. To increase the chances of enrichment of a specific anammox species that was adapted to low temperatures, sludge from the Haarlo WWTP (sample #5) was also added to the reactor, as it contained three different types (*Brocadia*-, *Kuenenia*- and *Scalindua*-like) of anammox bacteria as detected by clone libraries. This combined inoculum contained 0.33 g VSS of Haarlo sludge (#5) and 0.41 g VSS of Lichtenvoorde (#7) sludge.

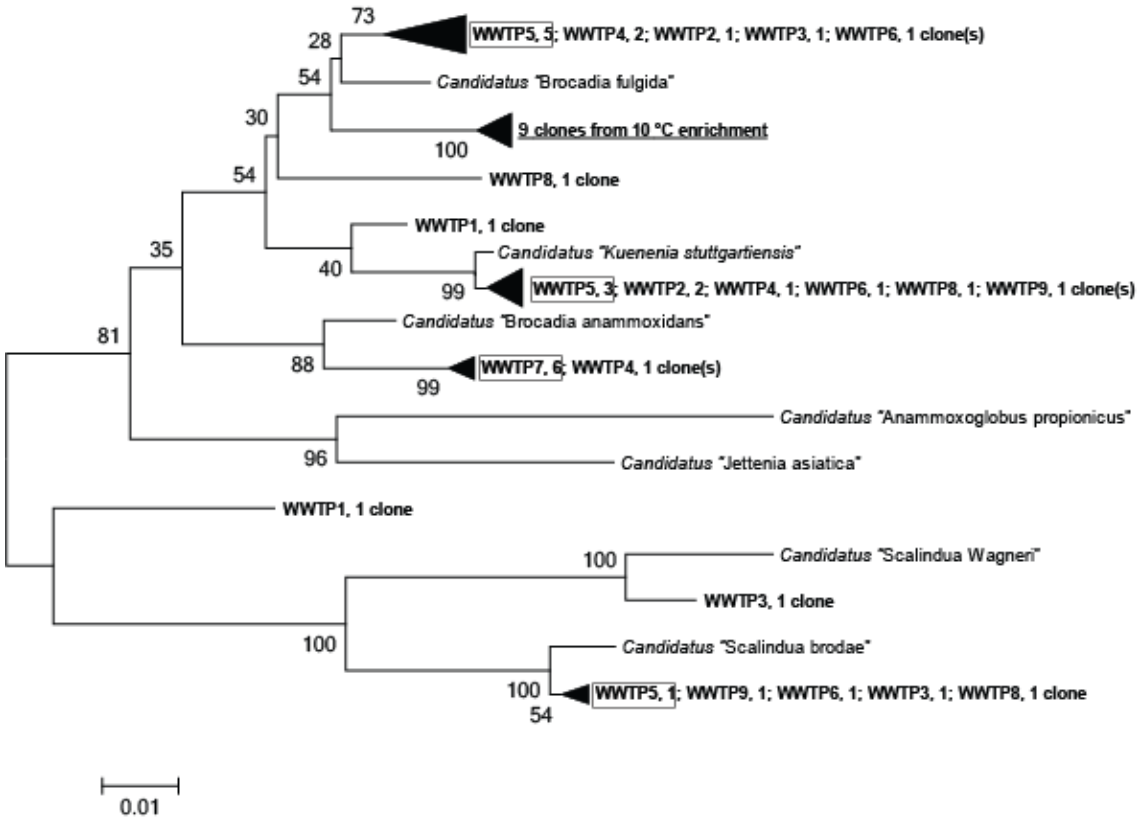
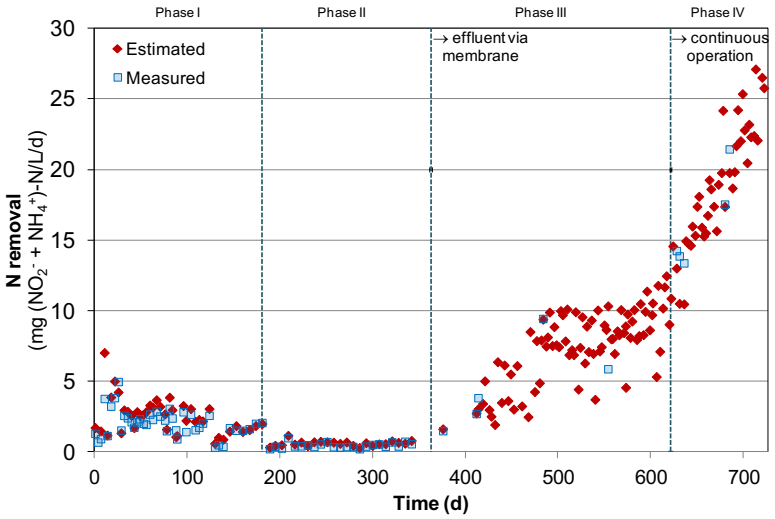


Figure 2 Phylogenetic tree showing the relationship of the 16S rRNA gene clones from sludge samples from 9 different waste water treatment plants (WWTP1…9) and the biomass from the 10°C enrichment reactor after 568 days of operation (10°C enrichment), to known anammox bacteria. WWTP5 and 7 (framed in the tree) were selected for inoculating the enrichment reactor. The (synthetic) influent of the enrichment reactor contained 61 mg (NH_4^+ + NO_2^-)-N/L and 10 vol% of stabilised and filtered effluent from an anaerobic reactor.

3.2 Anammox enrichment at 10°C

The enrichment of anammox bacteria from WWTP sludge resulted in a nitrogen removal rate (NRR) of up to 27 mg N/(L d) after 722 days, as is shown in Fig. 3. During the first 181 days of enrichment (phase I), the NRR was gradually decreasing, which can be explained by a gradual decrease in heterotrophic denitrification. Dissolved organic carbon present in the inoculum and produced by slow decay of organic material

and cellular biomass that was present in the inoculum were both available to drive heterotrophic denitrification. Once the organic material was depleted, nitrogen removal must take place autotrophically. The following period of day 181–363 (phase II) was marked by a very slow increase in NRR, from 0.2 to 0.7 mg N/(L d) and a very low biomass concentration of 0.07 g VSS/L. With this increase in activity, it was clear that anammox bacteria could grow in the system, which likely were an anammox species that was best adapted to 10 °C. To ensure full retention of the enriched anammox biomass, a membrane for effluent withdrawal was placed in the reactor on day 363 (phase III). This resulted in a clear and rapid increase in NRR up to 27 mg N/(L d). The conversion ratios shown in Table 3 are close to the anammox stoichiometry, again confirming that nitrogen removal was indeed achieved by anammox bacteria. Nitrate production is inevitably a result of growth of anammox bacteria, as part of the nitrite is oxidised to nitrate to provide electrons for carbon fixation. According to the anammox stoichiometry at mesophilic temperatures, this nitrate production is 0.26 mol NO_3^- per mol of NH_4^+ converted by anammox bacteria. In contrast to Hu et al. (2013a), the growth-related nitrate production by anammox bacteria was not below 0.26, indicating that biomass yield remained the same at this low temperature. Biomass concentration increased in phase IV from 0.43 g VS/L (day 631) to 0.51 g VS/L (day 722); using the total amount of (NH_4^+ + NO_2^-)-N converted in this period (7.3 g N), a biomass yield of 0.046 g VS/g N converted was calculated (assuming that the increase in VSS was only due to growth of anammox bacteria). This is indeed close to the stoichiometric anammox biomass yield of 0.050 g biomass/g N converted reported by Strous et al. (1999). Based on the exponential increase in activity between days 599 and 685 a maximum net growth rate of 0.011 d^{-1} at 10 °C was calculated.



284

285 **Figure 3** Nitrogen ($NH_4^+ + NO_2^-$) removal rate in the anammox enrichment reactor
286 operated at 10°C. The (synthetic) influent contained 61 mg ($NH_4^+ + NO_2^-$)-N/L and 10
287 vol% of stabilised and filtered effluent from an anaerobic reactor. Estimated rate was
288 based on measured nitrite conversion and anammox stoichiometry, for the measured
289 rate both ammonium and nitrite conversion were measured. The reactor was initially
290 operated as an SBR (phases I-III) and a membrane was used for full anammox biomass
291 retention from day 363 (phase III); continuous operation started on day 622 (phase IV).

292

293 **Table 3** Measured nitrogen removal activities and the conversion ratios of nitrite, nitrate
294 and ammonium in the anammox enrichment reactor at 10°C. Anammox stoichiometry
295 from Strous et al., 1999.

Time	Volumetric activity	Specific activity	NO_2/NH_4	NO_3/NH_4
(d)	mg (NO_2+NH_4)-N/(L·d)	mg (NO_2+NH_4)-N/(g VS·d)	(-)	(-)
181-363	0.2-0.7	0.7-10 [a]	-	-
412	2.7	41	1.36	-0.40
484	9.4	44	1.31	-0.09
554	5.9	n/a	1.61	-0.39
628	14.2	30	1.08	-0.26
680	17.5	41	1.29	-0.59

	anammox stoichiometry	*1.32*	*-0.26*

[a] 4 measurements, per g VSS

3.3 Characterisation enriched anammox biomass

The enriched biomass consisted of small sludge flocs (Fig. S1). Phylogenetic analysis on a sample from the enrichment reactor on day 568 revealed that the dominant species was 96% similar to *Candidatus Brocadia fulgida* (as shown in Fig. 2). On day 727, qPCR analysis showed that the anammox cell count was $(1.06 \pm 0.3) \times 10^7$ cells/mL. At that moment the sludge concentration was 0.5 g VS/L, resulting in $(2.12 \pm 0.6) \times 10^{10}$ cells/g VS. The specific activity of the enriched anammox biomass was 30–44 mg N/(g VS d) at 10 °C, as shown in Table 3. This is two times higher than a previously reported specific activity from a batch test at 10 °C, which was < 20 mg/(g VSS d) for sludge from a reactor operated at 30 °C (Dosta et al., 2008). Enrichment of anammox biomass at a low temperature may, therefore, be considered as beneficial for achieving a high specific activity. However, similar high specific activities may also be achieved after long-term adaptation of mesophilically grown anammox to 10 °C, which was not investigated in this research. Hu et al. (2013a) did report an adaptation over a period of 120 days at 12 °C, and showed that the optimum biomass specific activity at 12 °C was similar to the optimum specific activity at the start temperature of 30 °C. A batch experiment showed a high specific activity of 36 mg N/(g $VS_{anammox}$ d) at 10 °C (Hu et al., 2013a, using 0.6 g protein/g VS). Interestingly, Hu et al. (2013a) also found that *Ca. Brocadia fulgida* was the dominant species, as did Winkler et al. (2012) in an 18 °C reactor, inoculated with biomass from a 30 °C anammox reactor and receiving an influent containing ammonium and acetate. This might suggest that *Ca. Brocadia fulgida* may have a competitive advantage at low

320 temperatures. To confirm this, more low temperature anammox reactors should be
321 analysed for its anammox species and the mechanism(s) behind this advantage should
322 be elucidated.
323
324 *3.4 Temperature dependency*
325 Short-term experiments with the whole reactor and in batch incubations, indicated that
326 the optimum temperature of the enriched anammox species was between 20 and 30 °C
327 (Fig. 4). This is 10–15 °C lower than the optimum for anammox species used for
328 nitrogen removal at higher temperature, yet higher than the optima of anammox species
329 obtained from saline environments (see Table 4). The enriched anammox bacteria in the
330 current study did not appear to be psychrophilic, but more likely low temperature
331 adapted mesophiles. Hu et al. (2013a) showed that the temperature optimum of 12 °C
332 adapted anammox was also around 25 °C, whilst a higher optimum of around 35 °C was
333 found for biomass from a 30 °C reactor. In the temperature range of 5 to 17 °C, an
334 apparent activation energy of 66 kJ/mol was calculated. This is in the range of
335 activation energies of 63–70 kJ/mol determined for anammox biomass enriched at
336 temperatures ⩾20 °C (Hendrickx et al., 2012).
337
338 **Table 4** Temperature optima of anammox bacteria from low temperature natural
339 environments and from lab reactors.

Species	$T_{optimum}$ (°C)	Biomass source	T (°C)	Reference
n.d. (not determined)	15	environment	4 – 6	Dalsgaard and Thamdrup, 2002
n.d.	12	environment	-1.7 – 4	Rysgaard et al., 2004
Kuenenia stuttgartiensis	35 – 40	synthetic		Dosta et al., 2008
n.d.	37	leachate/synth.		Egli et al., 2001

several	37			Isaka et al., 2008
Ca. Brocadia fulgida	n.d.	30°C anammox reactor	18	Winkler et al., 2011
Ca. Brocadia fulgida	35	30°C anammox reactor	30	Hu et al., 2013a
Ca. Brocadia fulgida	25	30°C anammox reactor	12	Hu et al., 2013a
Ca. Brocadia fulgida	20 – 30	enrichment WWTP sludge	10	this study

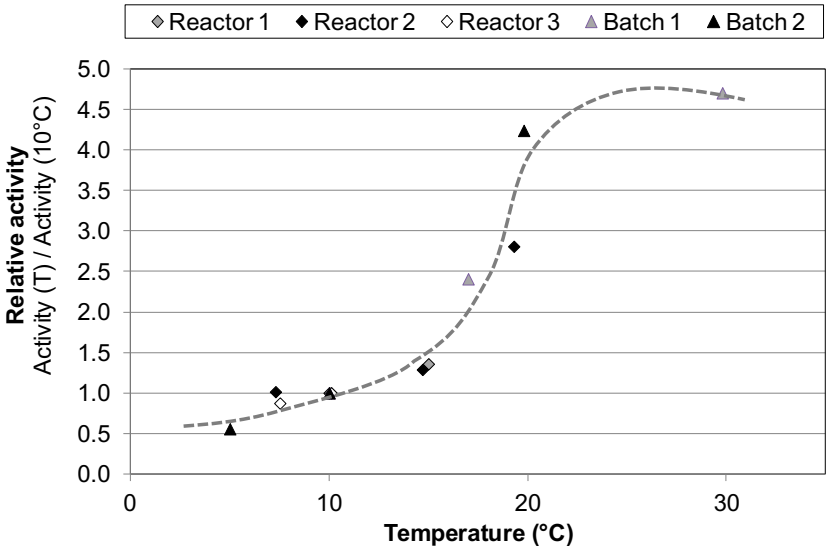


Figure 4 Volumetric activites at 5-30°C relative to the activity at the enrichment temperature of 10°C. Series of tests were performed with the whole reactor (diamonds) and in batch experiments (triangles). The broken line indicates the trend of the effect of temperature on the relative activity.

3.5 Low temperature application of anammox

The present study and previous studies have shown that growth of anammox at temperatures of 10–20 °C and at a low nitrogen concentration is feasible (Hu et al., 2013a, Hendrickx et al., 2012, Osaka et al., 2012). Growth of anammox bacteria at such low temperatures is a prerequisite for full-scale application, as it avoids the need to continuously inoculate with large amounts of new anammox bacteria. A next challenge

for full scale application is the production of nitrite, whilst preventing full nitrification to nitrate. At temperatures below 15–20 °C, the undesired nitrite oxidising bacteria (NOB) have a higher maximum growth rate than the required ammonium oxidising bacteria (AOB) (e.g. de Clippeleir et al., 2013). Achieving efficient retention of anammox bacteria and AOB, whilst preventing (excessive) NOB growth will prove to be a challenge, as shown by De Clippeleir et al. (2013). They operated a reactor with anammox bacteria and nitrifiers at a temperature as low as 14 °C, but reported an increase in NOB activity from 0 to 85 mg N/(L d) as temperature decreased from 22 to 14 °C. Hu et al. (2013a) showed a very efficient selection for AOB over NOB, in a nitritation-anammox reactor operated at 12 °C. However, this reactor was stoichiometrically fed with oxygen, and no NOB could be detected in the reactor. Stoichiometrically feeding oxygen will be difficult at full scale, as the oxygen availability will locally be higher at the point(s) of air inlet. Nonetheless, anammox exists and thrives in natural environments (e.g. in oxygen minimum zones) where the necessary nitrite is formed by AOB or AOA (ammonium oxidising archea) (Lam and Kuypers, 2011). This shows that even at temperatures well below 10 °C anammox and AOB can compete with NOB. In such oxygen minimum zones there is a limitation by oxygen that favours the selection of AOB, which also results in low specific conversion rates. Achieving a higher (specific) AOB rate will require a higher dissolved oxygen concentration and, therefore, strict control of sludge retention times of the different bacteria, so that the undesired NOB can still be outcompeted.

A specific activity of 30–44 mg N/(g VS d) at 10 °C was found for the enriched anammox biomass. For application in sewage treatment, a volumetric N loading of about 150 mg N/(L d) is required, when assuming a typical HRT of 10 h and an influent N concentration of 60 mg N/L (Metcalf and Eddy, 2004). This would mean that an

anammox biomass concentration of about 3–5 kg VS/m^3 is required at a waste water temperature of 10 °C. As previously discussed (e.g. Hendrickx et al., 2012), application of the anammox process at low temperature is most likely to be successful in a single reactor containing both anammox bacteria and AOB. Including the AOB, the total required biomass concentration in such a reactor will be 5–8 kg VS/m^3 (assuming the same specific activity for AOB and 57% nitritation by these AOB and assuming no retention of other micro-organisms). To maintain such a biomass concentration, traditional clarifiers at WWTPs will not be sufficient. Alternative retention techniques will be required, such as a membrane bioreactor (MBR), a rotating biological contactor (RBC) or a reactor with granular biomass. Of these, a granular biomass based reactor is likely to be most successful, since it allows for (1) a compact biomass retention device due to the high setting velocity of granular biomass, (2) selective washout of non-granular sludge, such as influent solids, NOB and heterotrophic growth on influent organic material, (3) lower effect of the granular biomass (compared to flocculent biomass) on the α-factor, which affects aeration energy input. The enrichment reactor in this study contained flocs. As the reactor was operated as a membrane bioreactor to retain all biomass, no selection pressure for growth in granules was applied. The feasibility of granulation of the anammox biomass at 10 °C still requires further research, as does preventing and/or removing growth of NOB and heterotrophs on the granule surface. Granulation at such low temperatures has been shown previously for aerobic biomass (e.g. Bao et al., 2009) and anaerobic biomass (e.g. Rebac et al., 1999). Furthermore, the enrichment time at 10 °C in this study was extremely long, mainly due to the initial selection period (363 days) caused by a very low fraction of anammox bacteria in the inoculum. With the availability of the enriched biomass, a further increase in the amount of biomass does not require this initial selection. Inoculation of a

low temperature anammox process may also be performed with granules from a mesophilic anammox reactor. The results of Hu et al. (2013a) present promising results for the fast adaptation to a lower temperature after decreasing the temperature from 30 to 12 °C. The feasibility of this has yet to be proven for granular sludge, as granule disintegration may occur as a result of lowering the temperature, as was previously observed for anaerobic granules (McKeown et al., 2009). Additionally, the lower measured nitrate production at 12 °C reported by Hu et al. (2013a) could be an indication that no/limited growth takes place after lowering the temperature, whereas the current study clearly showed that growth of the anammox species selected at 10 °C took place.

4. Conclusions

Enrichment of anammox species '*Ca. Brocadia fulgida*' was achieved at 10 °C and an influent nitrogen concentration of 61 mg N/L:

- Inoculum from selected municipal waste water treatment plants (WWTPs).
- Enriched biomass appeared to have an activity optimum between 20 and 30 °C, lower than the optimum for anammox enriched at higher temperatures.
- Biomass yield at 10 °C was 0.046 g VS/g N converted, similar to that at higher temperatures.
- Average biomass specific activity was 39 mg N/(g VS d) at 10 °C, a factor 2 higher than previously reported.
- Realistic required biomass concentrations for anammox application in water line of WWTPs.

Acknowledgements

Tim Hendrickx and Christel Kampman were supported by the Foundation for Applied Research (STW 07736). Ziye Hu and Boran Kartal were supported by European Research Council Advanced Grant (232937) and the Netherlands Organization for Scientific Research Grant (VENI, 863.11.003), respectively. Erwin van der Biezen is acknowledged for the molecular analyses of the WWTP samples. The authors thank Simon Piveteau and Yang Wang for their assistance in operation of the reactor. The authors thank the operators and technologists of the water boards of HHSK, HDSR, WSHD, WRIJ and Wetterskip Fryslan for providing the sludge samples and the process data of the WWTPs.

References

1. Abma, W.R., Schultz, C.E., Mulder, J.W., van de Star, W.R.L., Strous, M., Tokutomi, T., van Loosdrecht, M.C.M., 2007. Full-scale granular Anammox process. Water Science and Technology, 55(8-9) 27-33. DOI:10.2166/wst.2007.238
2. APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC.
3. Bao, R., Yu, S., Shi, W., Zhang, X., Wang, Y., 2009. Aerobic granules formation and nutrients removal characteristics in sequencing batch airlift reactor (SBAR) at low temperature. Journal of Hazardous Materials, 168, 1334-1340. DOI:10.1016/j.jhazmat.2009.03.020
4. Dalsgaard, T., Thamdrup, B., 2002. Factors controlling anaerobic ammonia oxidation with nitrite in marine sediments. Applied and Environmental Microbiology, 68(8), 3802-3808. DOI:10.1128/AEM.68.8.3802–3808.2002

5. de Clippeleir, H., Vlaeminck, S.E., de Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., Boon, N., 2013. One-stage partial nitritation/anammox at 15 °C on pretreated sewage: feasibility demonstration at lab-scale. Appl. Microbiol. Biotechnol., 97(23) 10199-10210. DOI:10.1007/s00253-013-4744-x
6. Dosta, J., Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Álvarez, J., Méndez, R., 2008. Short- and long-term effects of temperature on the Anammox Process. Journal of Hazardous Materials, 154, 688-693. DOI:10.1016/j.jhazmat.2007.10.082
7. Egli, K., Fanger, U., Alvarez, P.J.J., Siegrist, H., van der Meer, J.R., Zehnder, A.J.B., 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch. Microbiol., 175, 198-207. DOI:10.1007/s002030100255
8. Frijters, C.T.M.J., Silvius, M., Fischer, J., Haarhuis, R., Mulder, R., 2007. Full-scale applications for both COD and nutrient removal in a CIRCOX® airlift reactor. Water Science and Technology, 55(8-9), 107-114. DOI:10.2166/wst.2007.248
9. Harhangi, H.R., Le Roy, M., van Alen, T., Hu, B.-L., Groen, J., Kartal, B., Tringe, S.G., Quan, Z.-X., Jetten, M.S.M., Op den Camp, H.J.M., 2012. Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. Applied and Environmental Microbiology, 78, 752–758. DOI:10.1128/AEM.07113-11
10. Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., Buisman, C.J.N., 2012. Autotrophic nitrogen removal from low strength waste

478 water at low temperature. Water Research, 46, 2187-2193.
479 DOI:10.1016/j.watres.2012.01.037
480 11. Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M., Kruit, J.,
481 Jetten, M.S.M., Kartal, B., 2013a. Nitrogen removal by a nitritation-anammox
482 bioreactor at low temperature. Applied and Environmental Microbiology, 79(8),
483 2807-2812. DOI:10.1128/AEM.03987-12
484 12. Hu, Z., Lotti, T., van Loosdrecht, M.C.M., Kartal, B., 2013b. Nitrogen removal
485 with the anaerobic ammonium oxidation process. Biotechnol. Lett., 35(8), 1145-
486 1154. DOI:10.1007/s10529-013-1196-4
487 13. Isaka, K., Date, Y., Kimura, Y., Sumino, T., Tsuneda, S., 2008. Nitrogen
488 removal performance using anaerobic ammonium oxidation at low temperatures.
489 FEMS Microbiol. Lett., 282, 32-38. DOI: 10.1111/j.1574-6968.2008.01095.x
490 14. Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-
491 Roser, A., Koops, H.P.M, Wagner, M., 1998. Combined molecular and
492 conventional analyses of nitrifying bacterium diversity in activated sludge:
493 Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations.
494 Applied and Environmental Microbiology, 64, 3042–3051.
495 15. Kartal, B., Kuenen, J.G., van Loosdrecht, M.C.M., 2010. Sewage Treatment
496 with Anammox. Science, 238, 702-703. DOI:10.1126/science.1185941
497 16. Lam, P. Kuypers, M.M.M., 2011. Microbial nitrogen cycling processes in
498 oxygen minimum zones. Annual Review of Marine Science, 3, 317–345.
499 DOI:10.1146/annurev-marine-120709-142814
500 17. Luesken, F., van Alen, T.A., van der Biezen, E., Frijters, C., Toonen, G.,
501 Kampman, C., Hendrickx, T.L.G. Zeeman, G., Temmink, H., Strous, M., Op den
502 Camp, H.J.M., Jetten, M.S.M., 2011a. Diversity and enrichment of nitrite-

503 dependent anaerobic methane oxidizing bacteria from wastewater sludge,
504 Applied microbiology and biotechnology, 92(4), 845-854. DOI:10.1007/s00253-
505 011-3361-9
506 18. Luesken, F.A., Sánchez. J., van Alen, T.A., Sanabria, J., op den Camp, H.J.M.,
507 Jetten, M.S.M.,Kartal, B., 2011b. Simultaneous nitrite-dependent anaerobic
508 methane oxidation and ammonium oxidation. Applied and Environmental
509 Microbiology, 77(19) 6802-6807. DOI:10.1128/AEM.05539-11
510 19. Ma, B., Peng, Y., Zhang, S., Wang, J., Gan, Y., Chang, J., Wang, S., Wang S.,
511 Zhu, G., 2013. Performance of anammox UASB reactor treating low strength
512 wastewater under moderate and low temperatures. Bioresource Technology,
513 129, 606-611. DOI:10.1016/j.biortech.2012.11.025
514 20. McKeown, R.M., Scully, C., Mahony, T., Collins, G., O'Flaherty, V., 2009.
515 Long-term (1243 days), low-temperature (4–15 °C), anaerobic biotreatment of
516 acidified wastewaters: Bioprocess performance and physiological
517 characteristics. Water Research, 43, 1611-1620.
518 DOI:10.1016/j.watres.2009.01.015
519 21. Metcalf and Eddy, 2004. Waste water engineering, treatment disposal and reuse.
520 4th edition, McGraw-Hill, Singapore.
521 22. Neef, A., Amann, R., Schlesner, H., Schleifer, K.H., 1998. Monitoring a
522 widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-
523 targeted probes. Microbiology, 144, 3257–3266. DOI:10.1099/00221287-144-
524 12-3257
525 23. Osaka, T., Kimura, Y., Otsubo, Y., Suwa, Y., Tsuneda, S., Isaka, K., 2012.
526 Temperature dependence for anammox bacteria enriched from freshwater

527 sediments. Journal of Bioscience and Bioengineering, 114(4), 429-434.
528 DOI:10.1016/j.jbiosc.2012.05.003
529 24. Rebac, S., Gerbens, S., Lens, P., Van Lier, J.B., Stams, A.J.M., Keesman, K.J.,
530 Lettinga, G., 1999. Kinetics of fatty acid degradation by psychrophilically grown
531 anaerobic granular sludge. Bioresource Technology, 69(3), 241-248.
532 DOI:10.1016/S0960-8524(98)00195-3
533 25. Rysgaard, S., Glud, R.N., Risgaard-Petersen, N., Dalsgaard, T., 2004.
534 Denitrification and anammox activity in Artic marine sediments. Limnol.
535 Oceanogr. 49(5), 1493-1502.
536 26. Schmidt, T.M., Delong, E.F., Pace, N.R., 1991. Analysis of a marine
537 picoplankton community by 16S rRNA gene cloning and sequencing. J.
538 Bacteriol., 173, 4371– 4378.
539 27. Siegrist, H., Salzgeber, D., Eugster, J., Joss, A., 2008. Anammox brings WWTP
540 closer to energy autarky due to increased biogas production and reduced aeration
541 energy for N-removal. Water Science and Technology 57(3), 383-388.
542 DOI:10.2166/wst.2008.048
543 28. Strous, M, Kuenen, J.G., Jetten, M.S.M., 1999. Key physiology of anaerobic
544 ammonium oxidation. Applied and Environmental Microbiology, 65 (7), 3248–
545 3250.
546 29. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011.
547 MEGA5: molecular evolutionary genetics analysis using maximum likelihood,
548 evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28,
549 2731–2739. DOI:10.1093/molbev/msr121
550 30. van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen,
551 J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-

552 organisms in a fluidized bed reactor. Microbiology, 142, 2187-2196.
553 DOI:10.1099/13500872-142-8-2187
554 31. Van de Vossenberg, J., Rattray, J. E., Geerts, W., Kartal, B., van Niftrik, L., van
555 Donselaar, E.G., Sinninghe Damsté, J.S., Strous, M., Jetten, M.S.M., 2008.
556 Enrichment and characterization of marine anammox bacteria associated with
557 global nitrogen gas production. Environmental Microbiology, Vol. 10(11), pp.
558 3120-3129. DOI:10.1111/j.1462-2920.2008.01643.x
559 32. Vázquez-Pádin, J.R., Fernández, I., Morales, N., Campos, J.L., Mosquera-
560 Corral, A.,Méndez, R., 2011. Autotrophic nitrogen removal at low temperature.
561 Water Science and Technology, 63(6), 1282-1288. DOI:10.2166/wst.2011.370
562 33. Winkler, M.-K.H., Kleerebezem, R., van Loosdrecht, M.C.M., 2011. Integration
563 of anammox into the aerobic granular sludge process for main stream
564 wastewater treatment at ambient temperatures. Water Research, 46, 136-144.
565 DOI:10.1016/j.watres.2011.10.034
566 34. Zhang, L., Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Wei
567 Guang, L., Buisman, C.J.N., 2012. The effect of sludge recirculation rate on a
568 UASB-digester treating domestic sewage at 15°C. Water Science and
569 Technology, 66(12), 2597-2603. DOI:10.2166/wst.2012.487
570
571
572
573
574
575
576

S1

Fig. S1. Biomass from the anammox enrichment reactor after 727 days of operation, consisting of small flocs.